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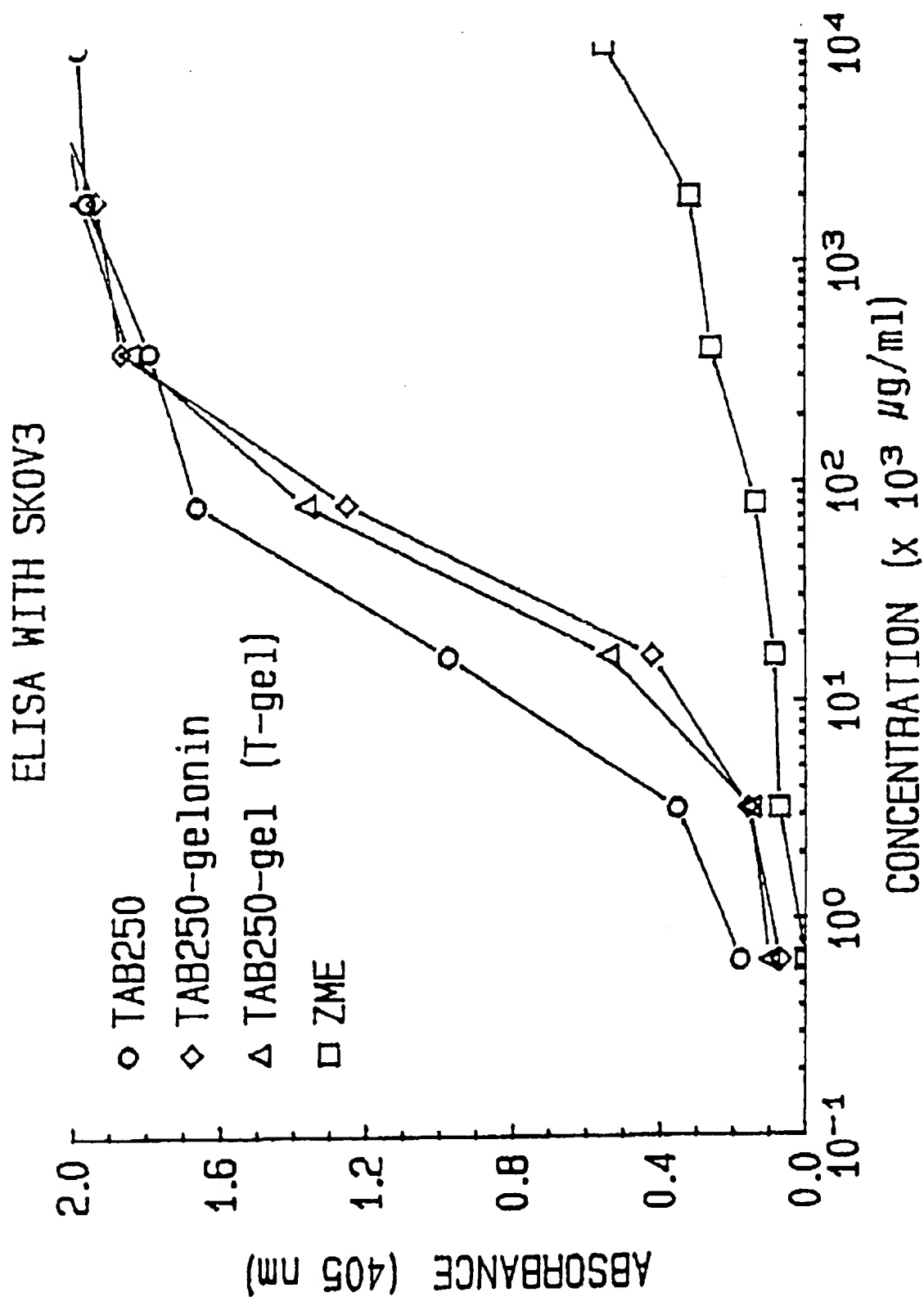


FIGURE 1

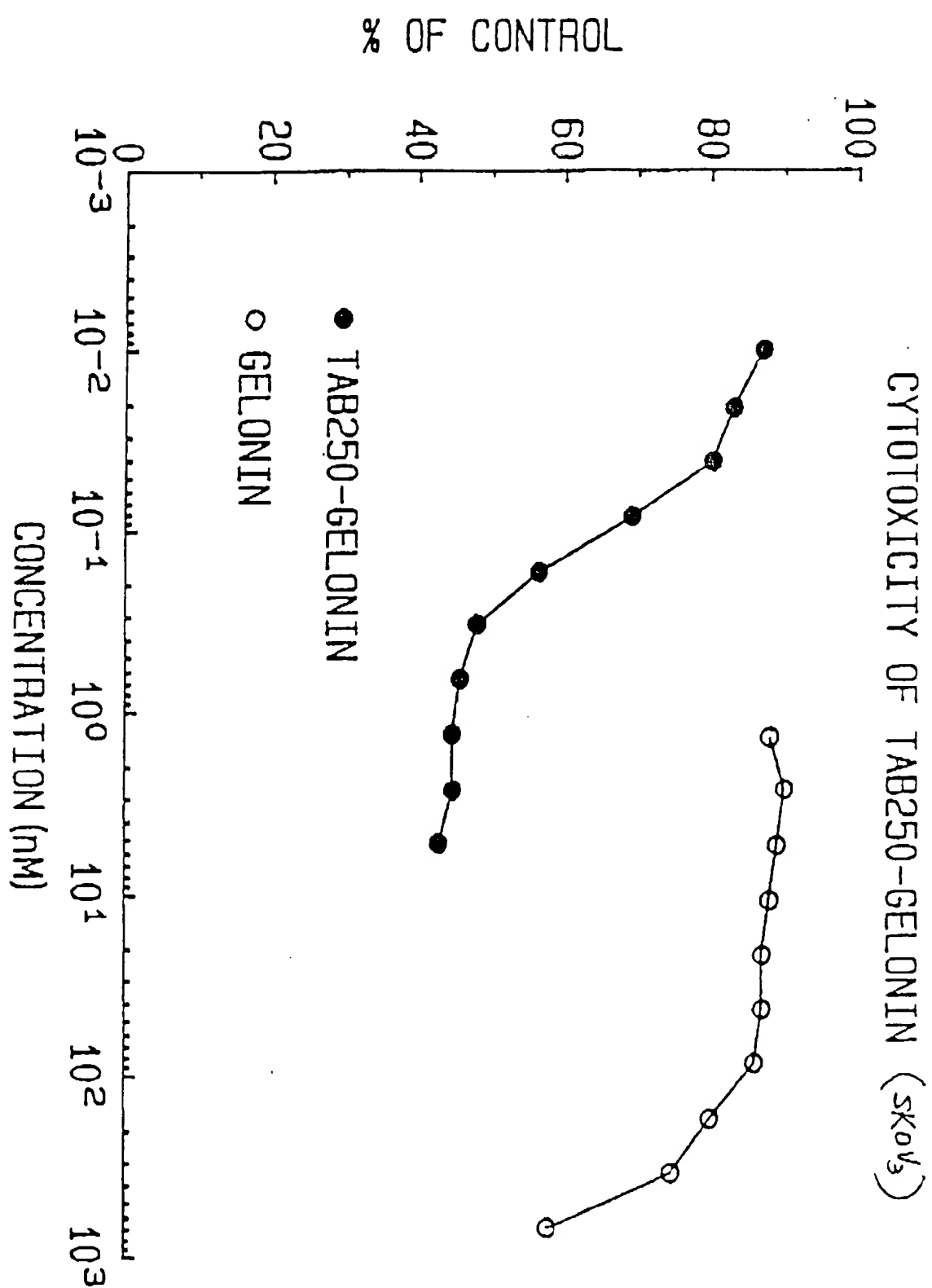


FIGURE 2

# COMPETITION OF RELEVANT VS IRRELEVANT ANTIBODY WITH CONJUGATE ON SKOV3

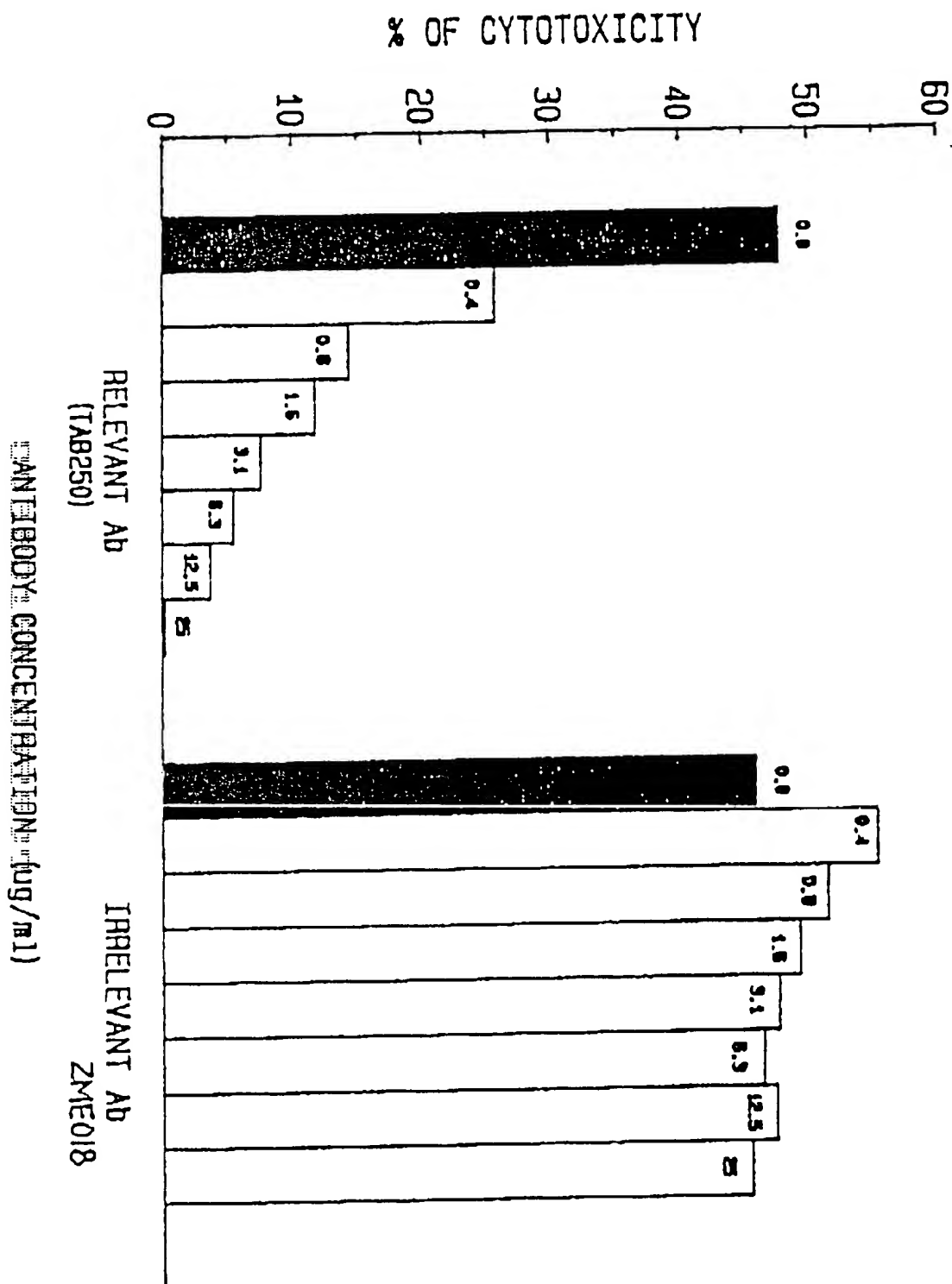


FIGURE 3

CPA

# 1Ab 250-Gelatin Dose Response on SKOV3 cells

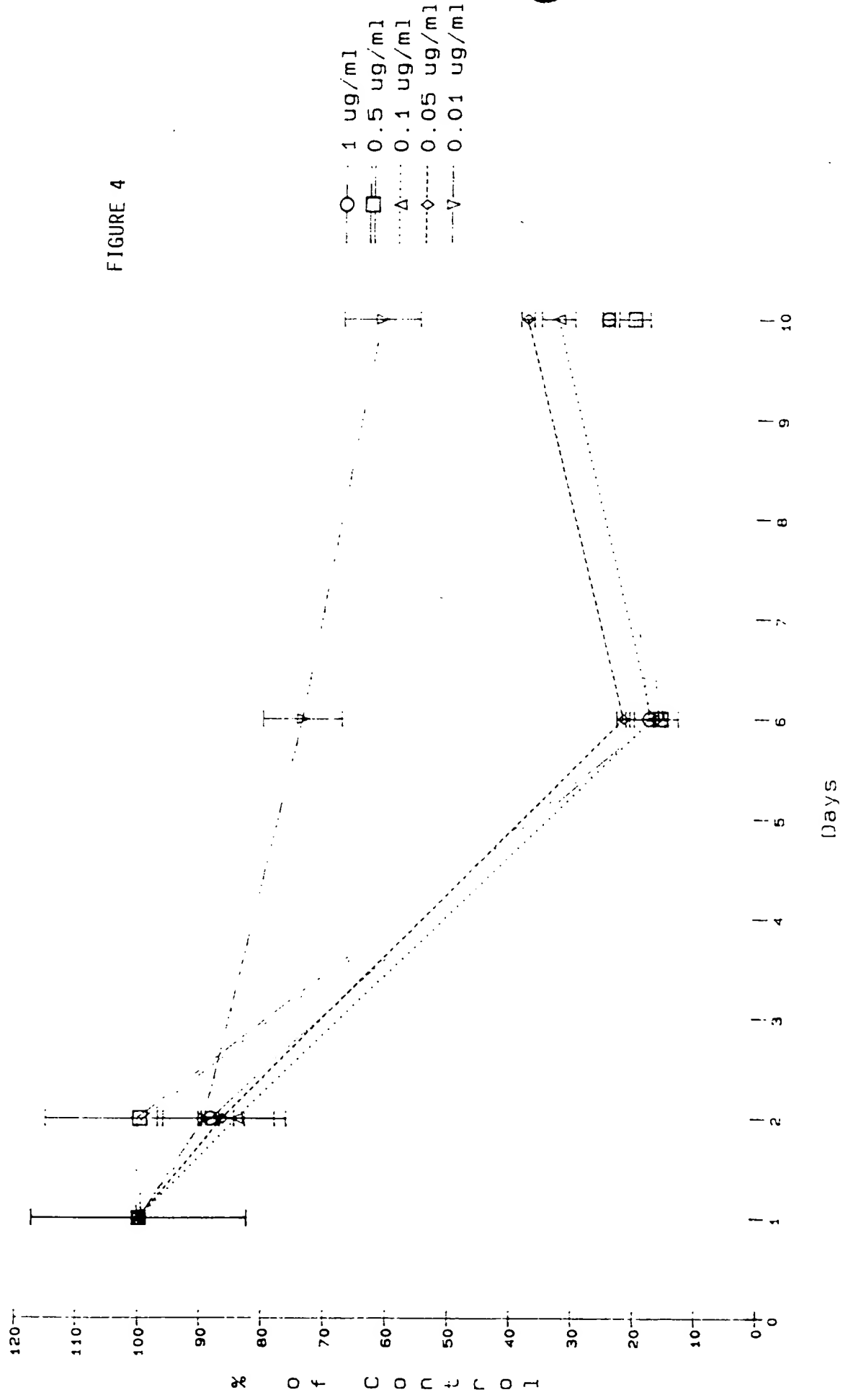
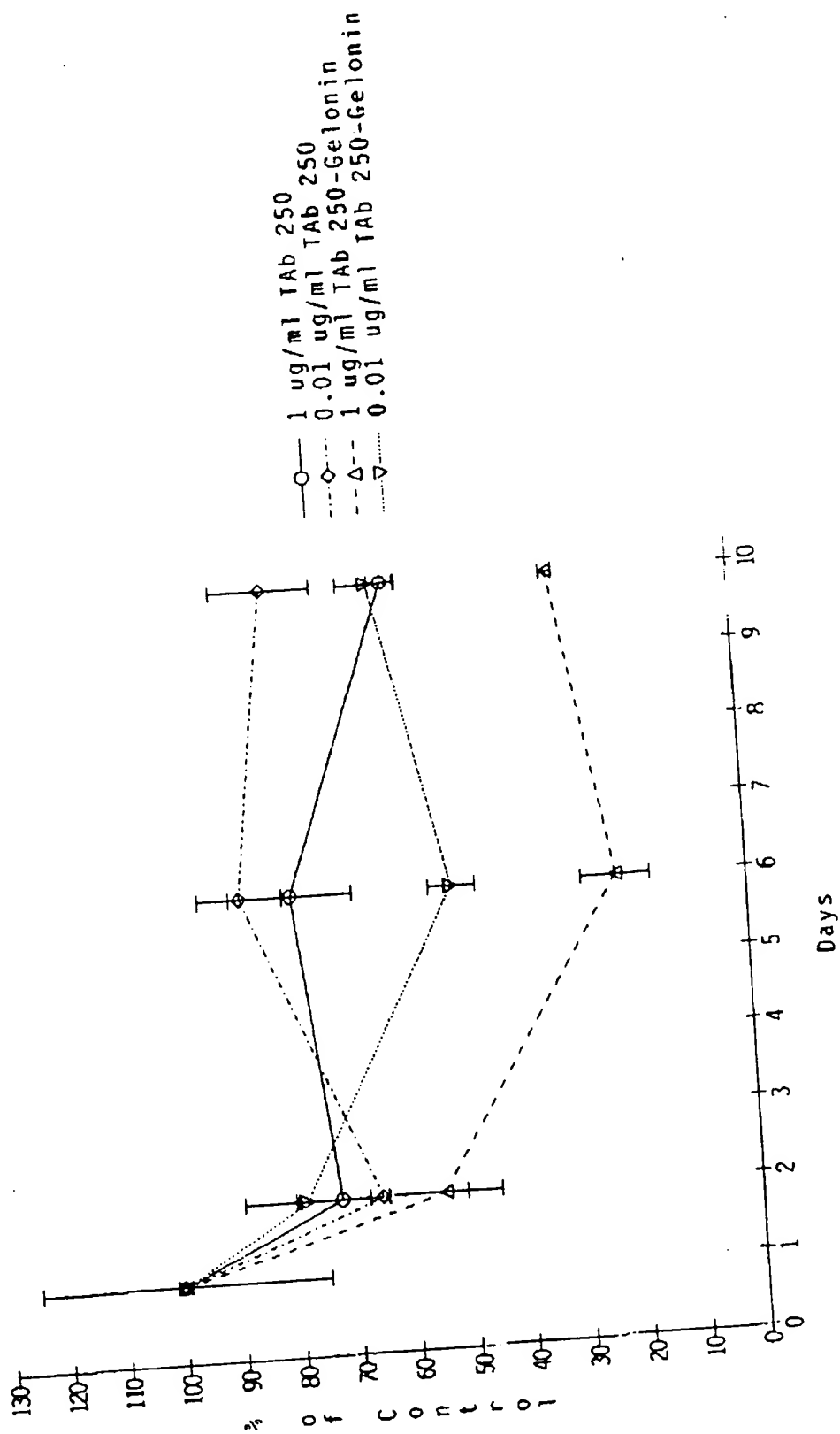


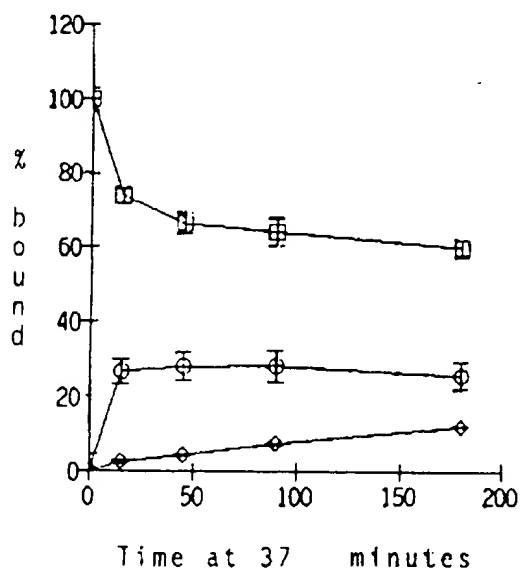
FIGURE 4

GCPA91991

CPA  
Effect of TAB 250 vs. TAB 250-Gelonin  
on SKOV3 cells

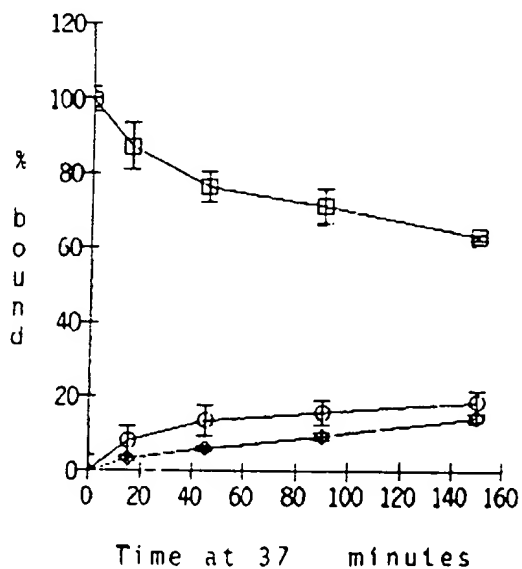


Internalization of Tab 250  
in SKBR3 cells



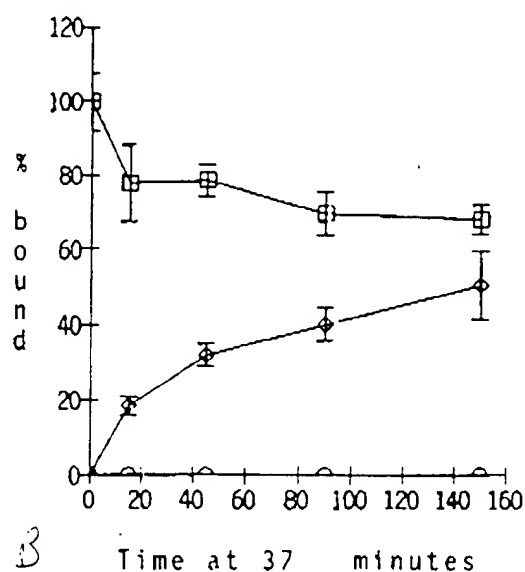
—○— internalized  
—◇— dissociated/recycled  
—□— surface bound

Internalization of Tab 250  
in SKOV3 cells



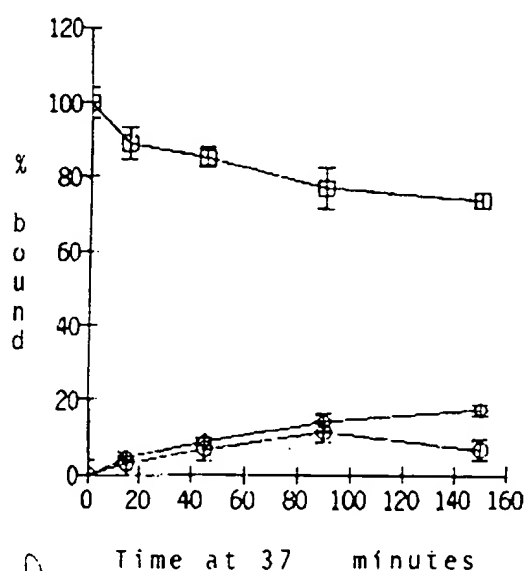
—○— internalized  
—◇— dissociated/recycled  
—□— surface bound

Internalization of Tab 250  
in MDA 231 cells



—◇— dissociated/recycled  
—□— surface bound  
—○— internalized

Internalization of Tab 250  
in MDA 453 cells



—○— internalized  
—◇— dissociated/recycled  
—□— surface bound

FIGURE 6

GM9591

TAb 250-Gelonin Dose Response  
MIT Assay

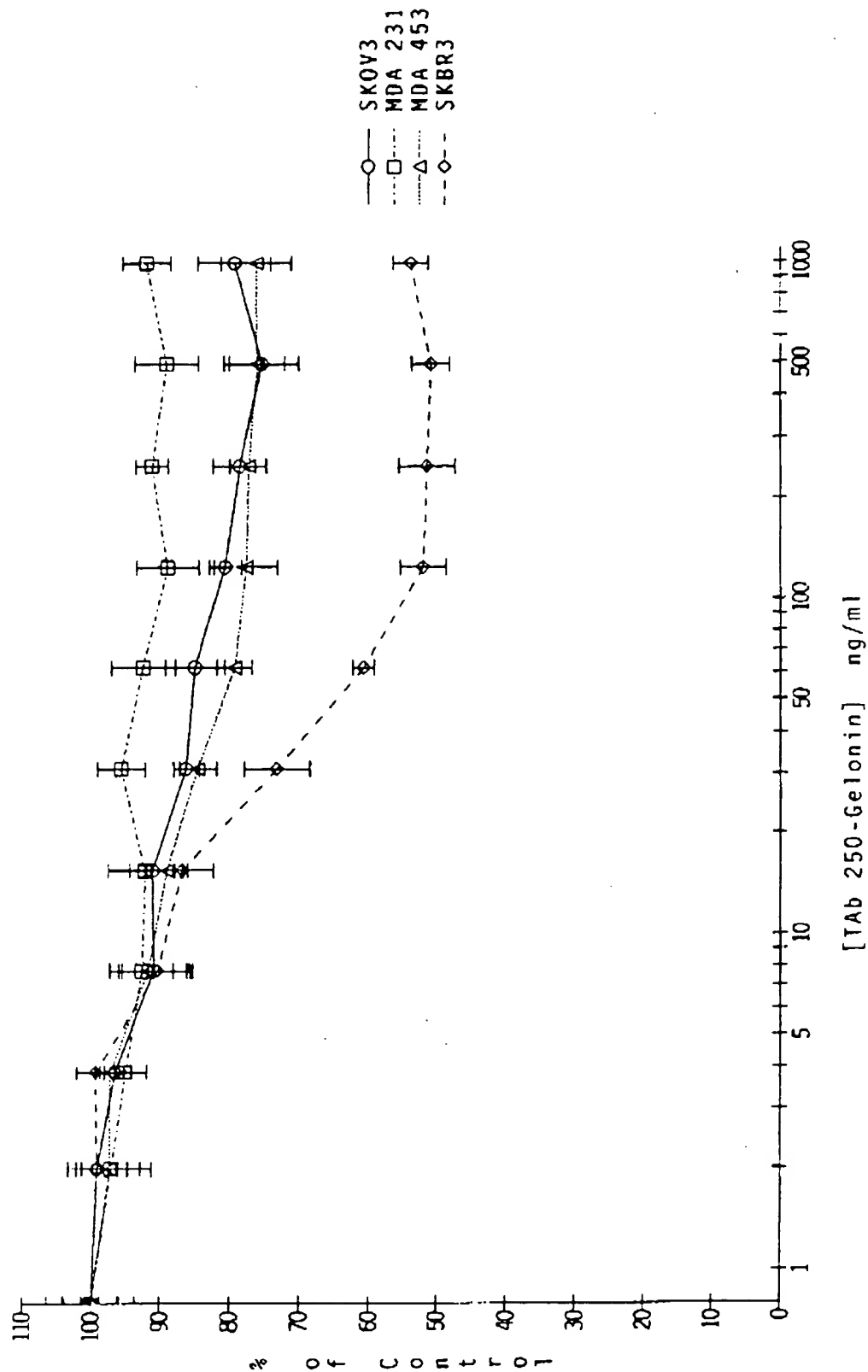


FIGURE 7

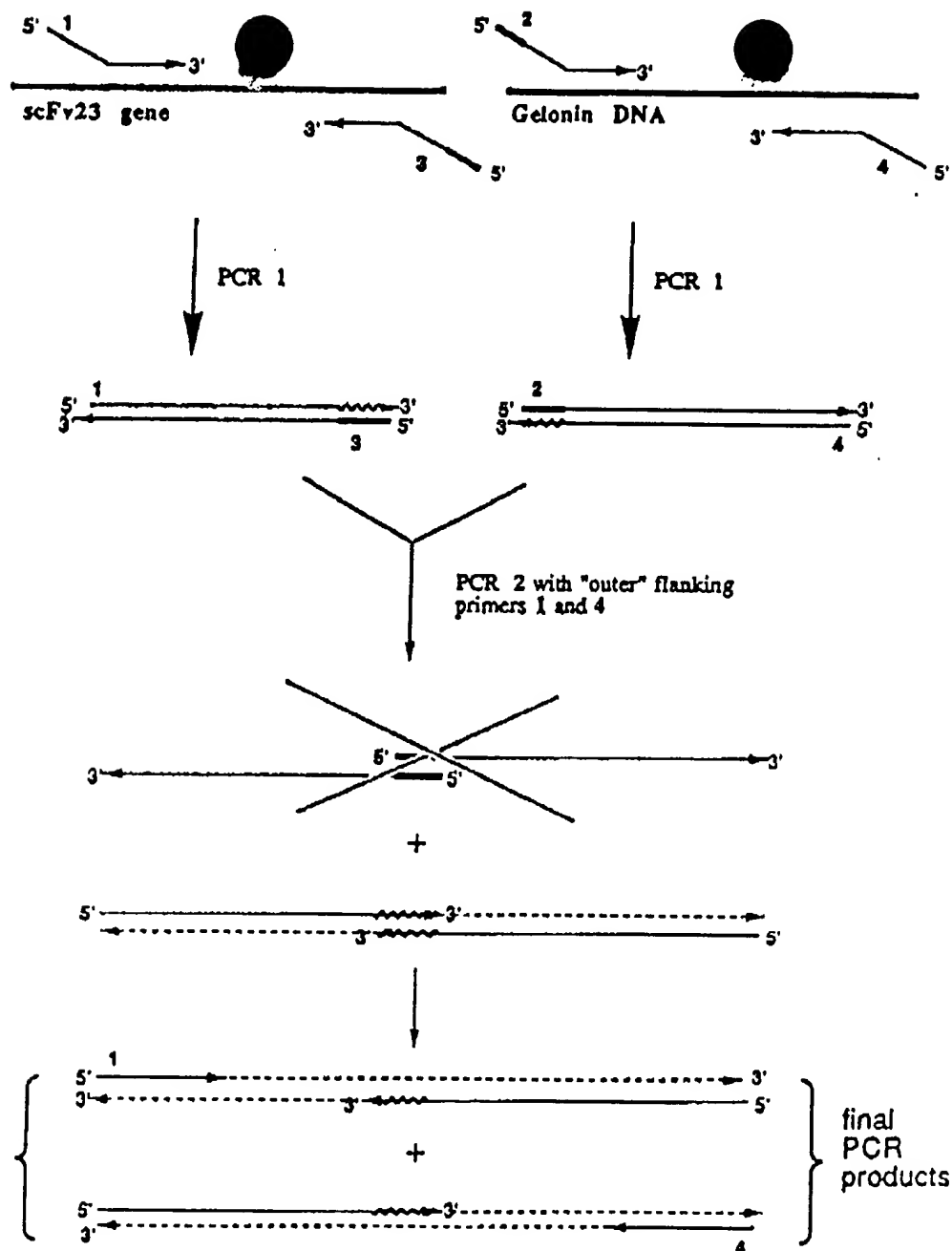
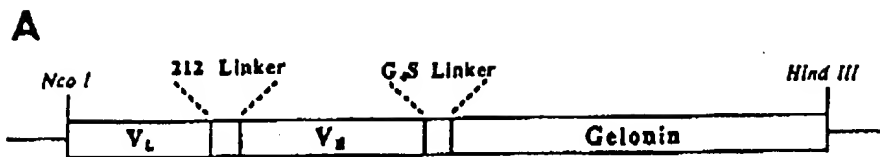


FIGURE 8

Figure 8: Construction of the scFv23 - Gelonin Immunotoxin gene. The first PCR syntheses were performed in separate tubes and one tenth of each of the reaction products were subsequently combined and used as templates for the second PCR (Davis et al. (1991) *BioTechnology* 9, 165-169). Nco I and Hind III restriction sites were included into the sequences of the "outer" flanking primers 1 and 4 for future cloning purposes. Homology between bases in the PCR primers 2 and 3 is indicated by the filled boxes at the 5' ends of each primer and represented DNA sequences encoding the Gly-Gly-Gly-Gly-Ser (G<sub>4</sub>S) linker between the antibody and gelonin fragments of the immunotoxin.





**B**

V<sub>L</sub> scFv23

CCC ATG GCG ATC TCT GAC CTC CAG CTG ACC CAG TCT CCA CCA ATC CTC TCT CCA TCT  
Pro Met Ala Met Ser Asp Val Glu Leu Thr Gln Ser Pro Ala Ile Leu Ser Ala Ser

LCDR1

CCA CCG CAG AAG CTC ACA ATC ACT TCC AAG GCG GCG CCA AAG GTA AAT TAC AAG CAG  
Pro Gly Glu Lys Val Thr Met Thr Cys Asn Ala Thr Pro Ser Val Ser Tyr Met His

LCDR2

TGG TAT CAG CAG AAG CCA GGA TCG TCC CCA AAA CTT TGG ATT TAT ACG ACA TGG AAC  
Trp Tyr Gln Glu Lys Pro Gly Ser Ser Pro Lys Pro Trp Ile Tyr Thr Thr Ser Asn

LCDR3

CTG GCT TCT GGA CTC CTT GCT CCG TTC ACT GCG GCG CCG TCT GCG ACC TCT TAC TCT  
Leu Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Gly Gly Ser Gly Thr Ser Tyr Ser

312 linker

CTC ACA CTC ACC ACA CTC GAG GCT GAA GAT GCT CCG ACT TAT TAC TCC GAG CAG TGG  
Leu Thr Val Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Glu Glu Thr

65S linker

ATT GCG AAG CCA CCG ACC TTC CCA CCG GCG TCC AAG CTC GAA ATA AAA GCG TCT ACC  
Ser Arg Ser Pro Pro Thr Phe Gly Gly Gly Ser Lys Leu Glu Ile Lys Gly Ser Thr

V<sub>H</sub> scFv23

TCT GGT TCT GGT AAA TCT TCT GAA GGT AAA GGT GTG CAG CTG CAG TCA CCA CCG  
Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Val Gln Leu Glu Glu Ser Gly Pro

LCDR1

CAC CTG CTC AAG CCG CCA CCG TCA ATC AAG ATA TCC TCC AAG ACT TCT GGT TAC TCA  
Glu Val Val Lys Pro Gly Gly Ser Met Lys Ile Ser Cys Lys Thr Ser Gly Tyr Ser

LCDR2

TTC ACT GCG CAC ACC ATG AAC TGG GTG AAG CAG ACC CAT CCA AAG AAC CTT GAG TCG  
Phe Thr Gly His Thr Met Asn Trp Val Lys Glu Ser His Gly Lys Asn Leu Glu Tyr

LCDR3

ATT CCA GGT ATT AAT GGT TAC AAT GGT GAT ACT AAT TAC AAT CAG AAT TTT AAG GCG  
Ile Gly Lys Ile Asn Pro Tyr Asn Gly Arg Thr Asn Tyr Asn Glu Lys Thr Lys Gly

LCDR4

AAG GCG ACA TTT ACT GTA CAG AAG TCG TCC ACC ACA CCG TAC ATG CAG CTC CTC AGT  
Lys Ala Thr Phe Thr Val Asp Lys Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser

LCDR5

CTG ACA TCT GAG CAG TCT CCA CTC TAT TAC TGT CCA ACG AAG GGT ACC GAG TGG TAC  
Leu Thr Ser Tyr Asp Ser Ala Val Tyr Tyr Cys Ala Arg Asn Val Thr Asn Thr Tyr

65S linker

TTC GAT GTC TTT GCG CCA GCG ACC ACC CTC ACC CTC TCG CTC TCC TCA GGT CCG GGT  
Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val Ser Val Ser Ser Gly Gly Gly

Gelonin

GAG TCC GGT GTA GAT ACC GGT ACC TTC ACC ACC AAA GCG GGT ACC TAT ATT ACC TAT  
Gly Ser Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr Tyr

CTT AAT TTC CTG AAC GAA CTG COT OTT AAA CTG AAA CCG GAA GGT AAC ACC CAT GCG  
Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly Asn Ser His Gly

ATC CCG CTG CTG CCG AAA GGT GAT GAC CCG GGT AAA TGC TTC CTG CTG GCG CTG  
Ile Pro Leu Leu Arg Lys Gly Asp Asp Pro Gly Lys Cys Phe Val Leu Val Ala Leu

ACC AAC GAT AAC CCG CAG CTG CCA GAA ATC CCA ATC GAT GGT ACC ACC CTG TAC GTA  
Ser Asn Asp Asn Gly Glu Leu Ala Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val

OTT GCG TAT CAG GTG COT AAC CCG ACC TAC TTC TTC AAA GAT GGT CCG GAT CCA GCG  
Val Gly Tyr Glu Val Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala

TAC GAA CCG CTG TTC AAA AAC ACC ATC AAA AAC CCG CTG CTG TTC GGT CCG AAA ACT  
Tyr Glu Gly Leu Phe Lys Asn Thr Ile Lys Asn Pro Leu Leu Phe Gly Gly Lys Thr

COT CTG CAG TTC GGT GCG ACC TAT CCG ACC CTG GAA GCG GAA AAA GCG TAC CCG CAA  
Arg Leu His Phe Gly Gly Ser Tyr Pro Ser Leu Glu Gly Gly Lys Ala Tyr Arg Glu

ACT ACC GAT CTC GGT ATC GAA CCG GTG CCG ATC CCG ATC AAA AAA CTG CAG GAA AAC  
Thr Thr Asp Leu Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn

GCG ATC GAC AAC TAC AAA CCG ACC CAA ATC CCG ACC TCT CTG CTG CTT CTG ATC CAG  
Ala Ile Asp Asn Tyr Lys Phe Thr Glu Ile Ala Ser Ser Leu Leu Val Val Ile Glu

ATG GTG ACC GAA CCG CCA CCG TTC ACC TTC ATC GAA AAC CAG ATT COT AAC AAC TTC  
Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln Ile Arg Asn Asn Phe

CAG CAG COT ATC CCG CCG CCG AAC AAC ACC ATC TCT CTG GAA AAC AAA TCC CCG AAA  
Glu Glu Arg Ile Arg Pro Ala Asn Asn Thr Ile Ser Leu Glu Asn Lys Trp Gly Lys

CTG ACC TTC CAG ATC CCG ACC ACC GGT GCG AAC GGT ATG TTC ACC GAA CCG CTG CAA  
Leu Ser Phe Glu Ile Arg Thr Ser Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu

CTG GAA CCG GCG AAC CCG AAA AAA TAC TAC CTC ACT CCG GTG GAT CAG GTG AAA CCG  
Leu Glu Arg Ala Asn Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro

AAA ATC CCA CTG CTG AAA TTC CTC CAG AAA GAC GAC CTG  
Lys Ile Ala Leu Leu Lys Phe Leu Glu Lys Asp Glu Leu

(SEQ ID No. 10)

(SEQ ID No. 11)

FIGURE 9

Figure 9: Configuration and Sequence of the scFv23 - Gelonin immunotoxin. (A) The single-chain antibody fragment was cloned in the V<sub>L</sub>-212 linker-V<sub>H</sub> orientation and was fused to the toxin gelonin by a short, nonstructured five amino acid peptide (G<sub>4</sub>S). (B) The DNA sequence encoding the immunotoxin is shown with the corresponding protein sequence. The six antibody complementarity determining regions (CDRs) and the 212 linker sequence between the V<sub>L</sub> and V<sub>H</sub> domains are highlighted; the G<sub>4</sub>S sequence linking the antibody and toxin fragments is italicized. DNA encoding the synthetic gene for gelonin (Rosenblum et al. *Lymphokine Cytokine Res.* in press) was used as a template for the gene fusion and, in addition, encoded the targeting signal KDEL (Lys-Asp-Glu-Leu) incorporated into the 3' end.

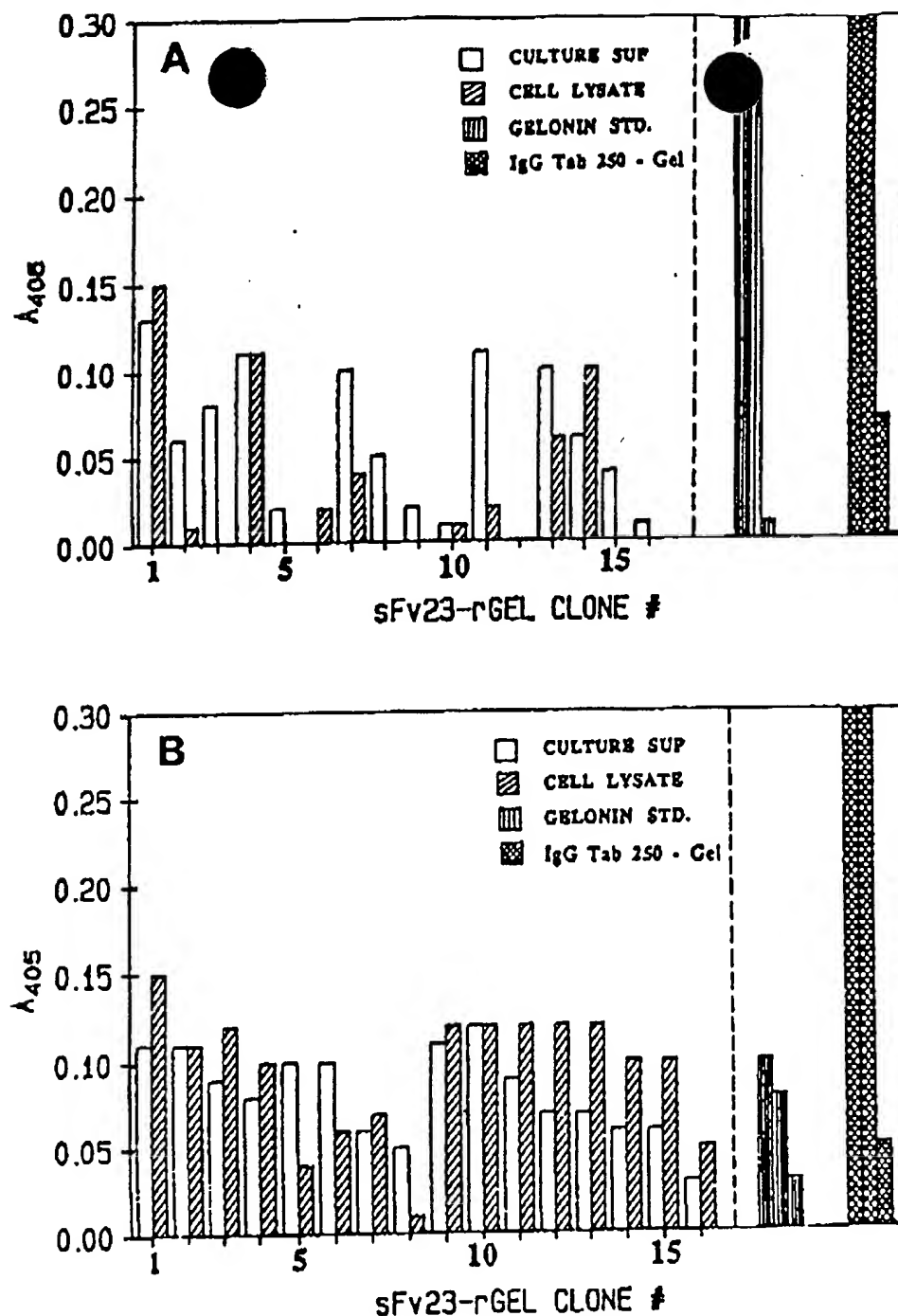


FIGURE 10

Figure 10: Binding analysis of sixteen individual scFv23 - Gelonin immunotoxin (IT) clones. Bacterial culture supernatants and periplasmic extracts of individually expressed ITs were added to wells of a 96-well ELISA plate coated with either (A) mouse *anti*-gelonin antibody 13A3, or (B)  $5 \times 10^4$  antigen-positive SK-OV-3 cells. Bound IT was detected with a rabbit *anti*-gelonin polyclonal antibody followed by addition of a horseradish peroxidase (HRPO) conjugate of a goat *anti*-rabbit IgG secondary antibody. Signals were developed with the HRPO substrate ABTS and measured at 405 nm. The negative and positive controls were free gelonin (at concentrations of 50  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , and 1  $\mu\text{g/mL}$ ) and a gelonin conjugate of Tab 250, a murine *anti*-HER-2/*neu* antibody (tested at 50 ng/mL, 10 ng/mL, and 1 ng/mL) respectively.

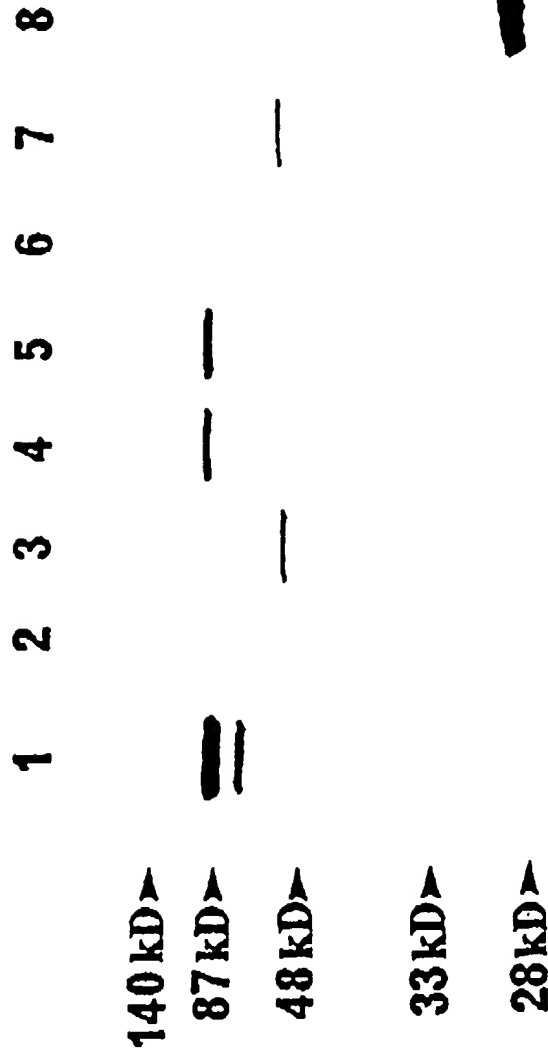


FIGURE 11

**Figure 11:** Western blot analysis of purified scFvZME-Gel and scFv-23-Gel proteins refolded and purified from insoluble inclusion bodies. Refolded GST fusions of the two immunotoxins were concentrated, bound to glutathione-agarose (GSH-ag) and digested with thrombin protease. The supernatant containing cleaved protein was retreated with GSH-ag to rebind any GST and immediately bound to Blue Sepharose (B.S.) resin. Immunotoxins were subsequently eluted with 2 M NaCl and dialyzed into TBS. Nitrocellulose filters used in the Western analysis were developed using the Amersham ECL system with an exposure time of 30 sec. Molecular weight standards are indicated on the left.

*Lane 1:* Refolded GST-fusion of scFvZME-Gel; *Lane 2:* Flowthrough after binding scFvZME-Gel to B.S.; *Lane 3:* scFvZME-Gel eluate from B.S.; *Lane 4:* GST-scFvZME-Gel molecular weight marker; *Lane 5:* Refolded GST-fusion of scFv-23-Gel; *Lane 6:* Flowthrough after binding scFv-23-Gel to B.S.; *Lane 7:* scFv-23-Gel eluate from B.S.; *Lane 8:* Gelonin standard (40 ng)

Figure 12

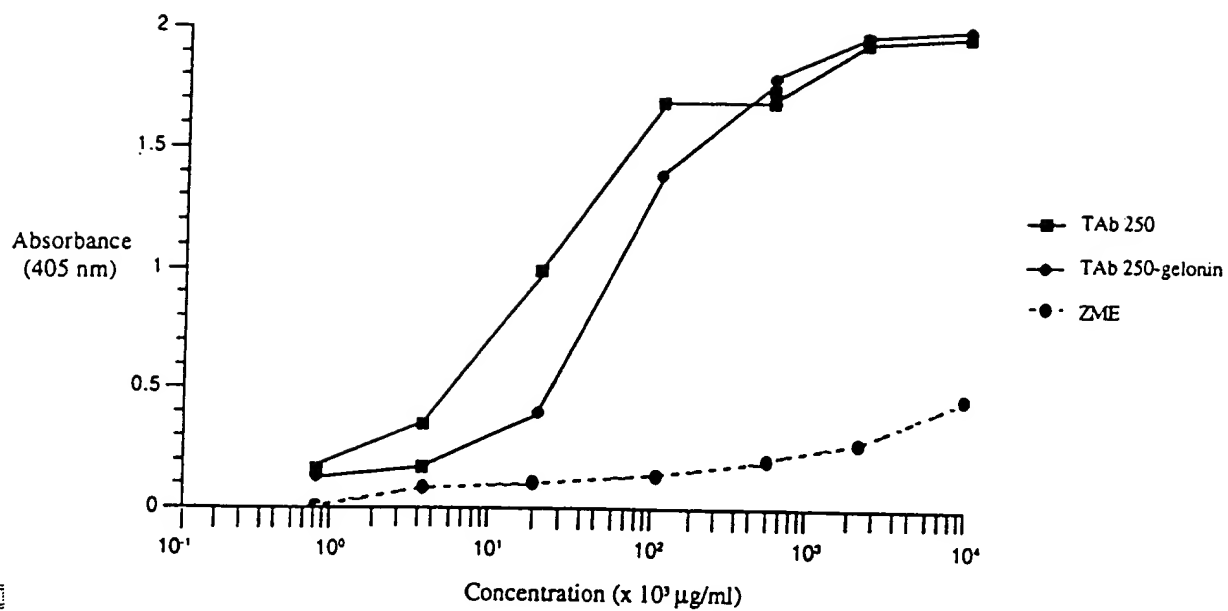


Figure 13

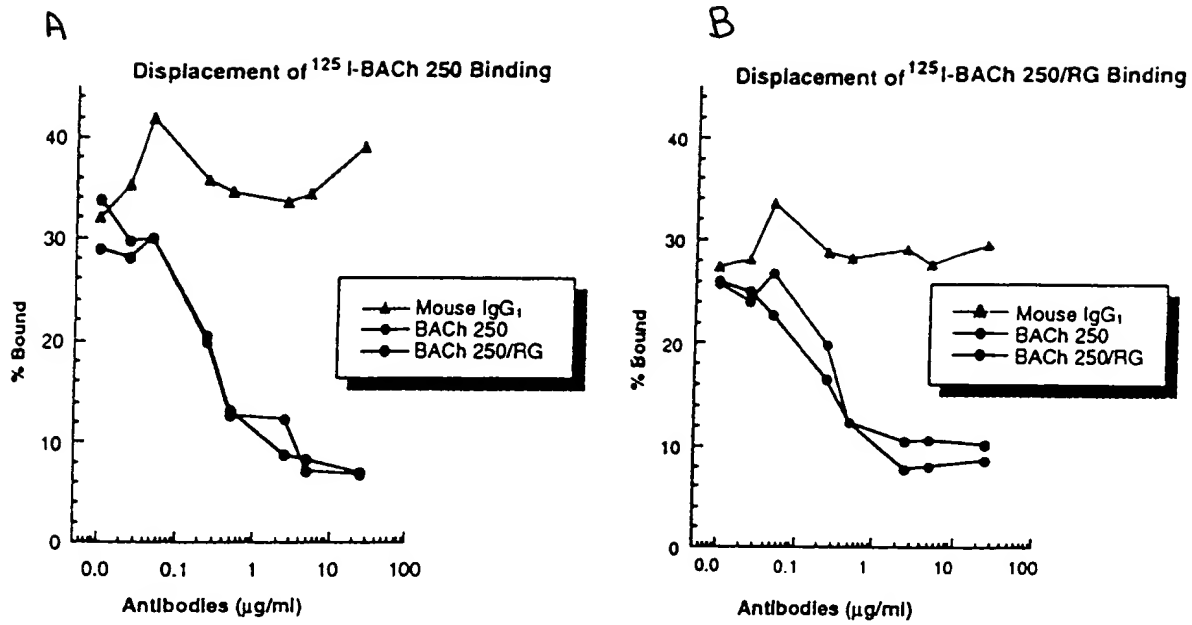
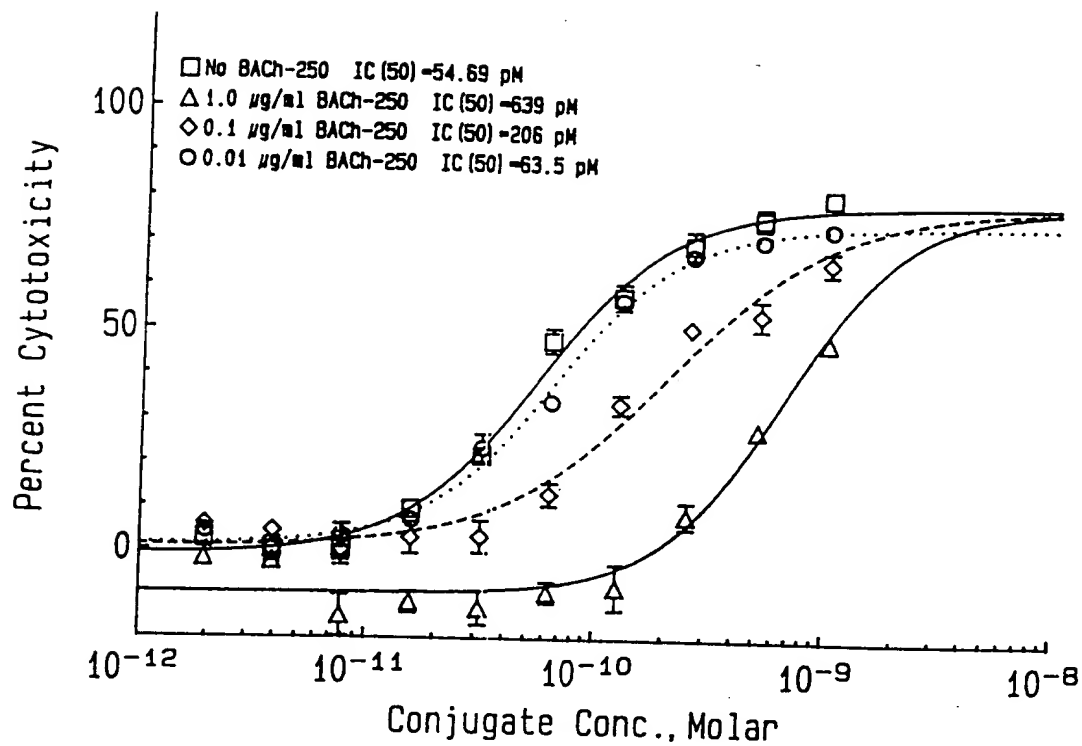


Figure 8 14



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Figure 4 15

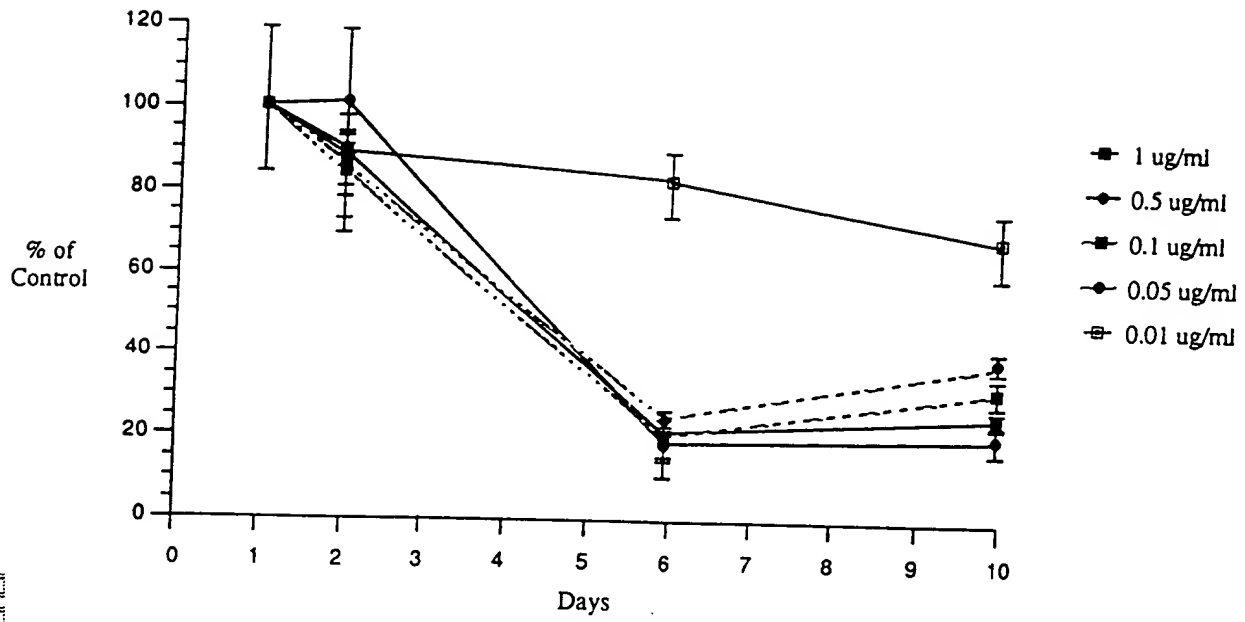
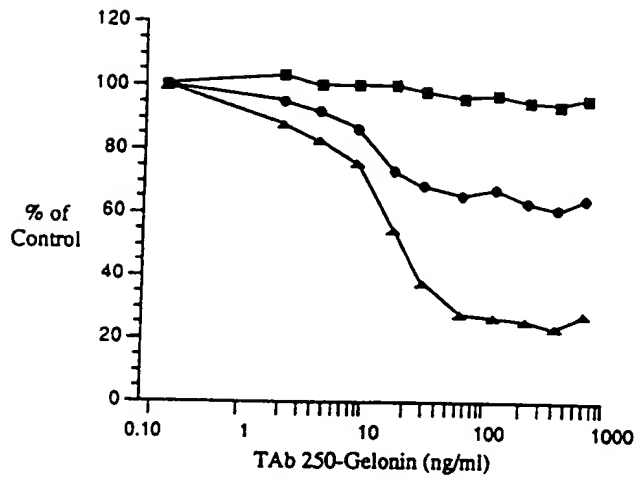
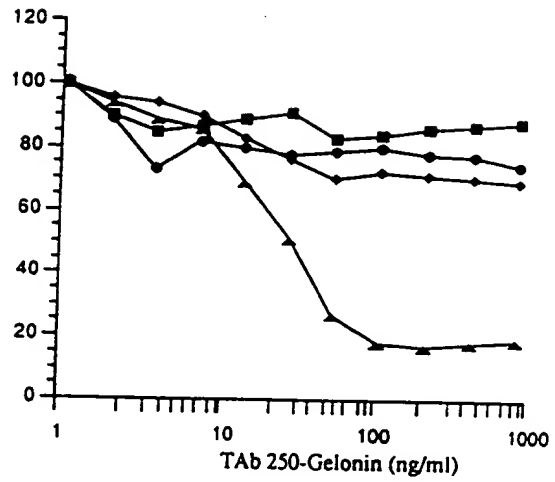


Figure 16



	# receptors
MDA-MB-231	16,000
SKOV-3	1,200,000
SKBR-3	4,000,000



	# receptors
MCF-7	78,000
OVCAR-3	150,000
MDA-MB-453	349,000
SKBR-3	4,000,000

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Figure 17

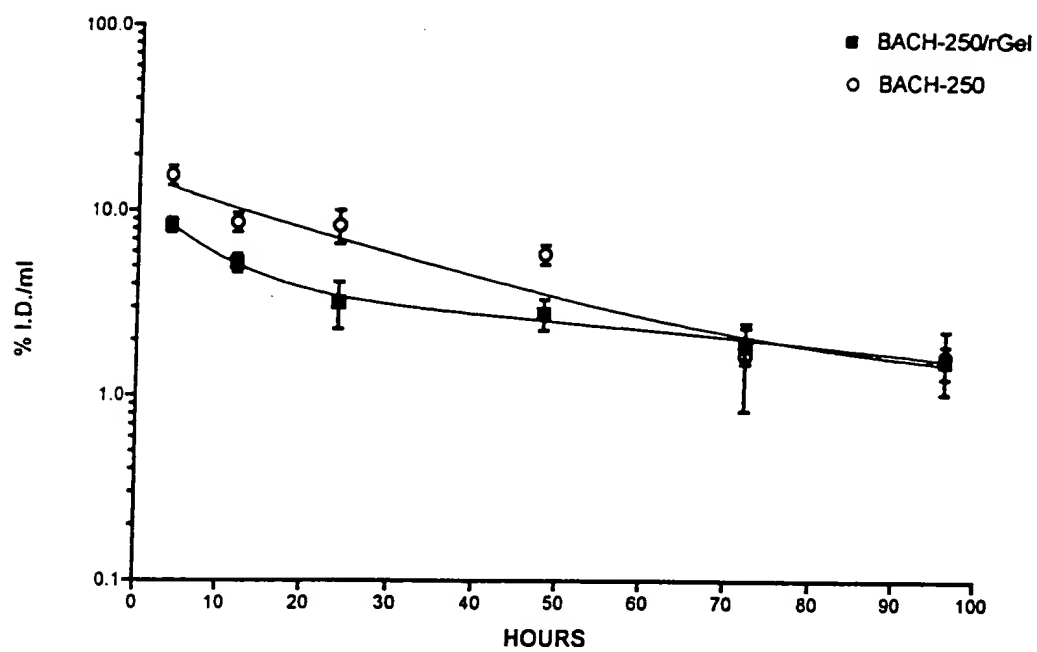


Figure 4 18

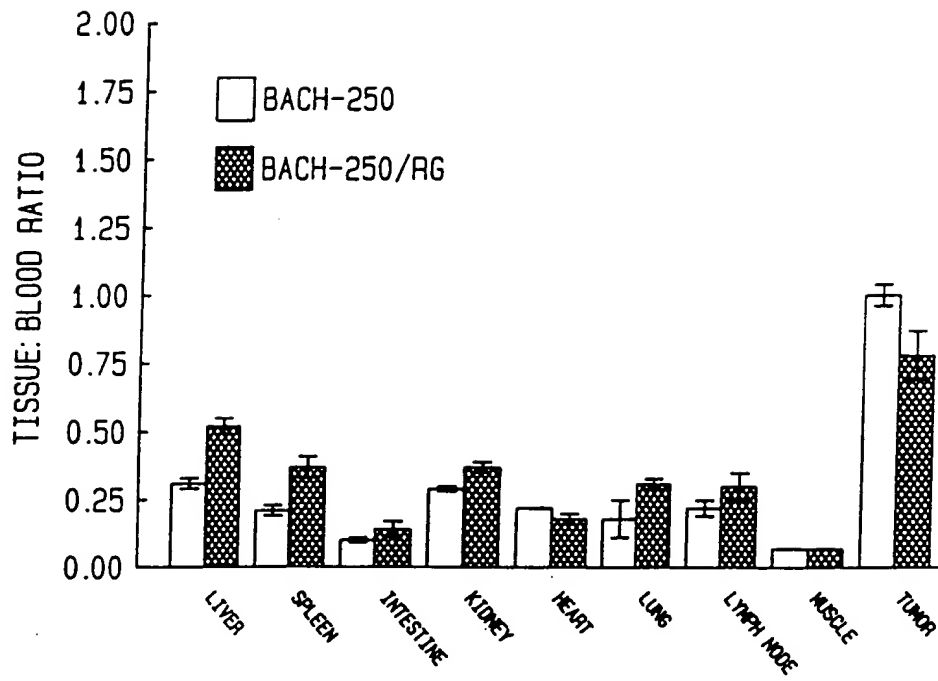


Figure 19

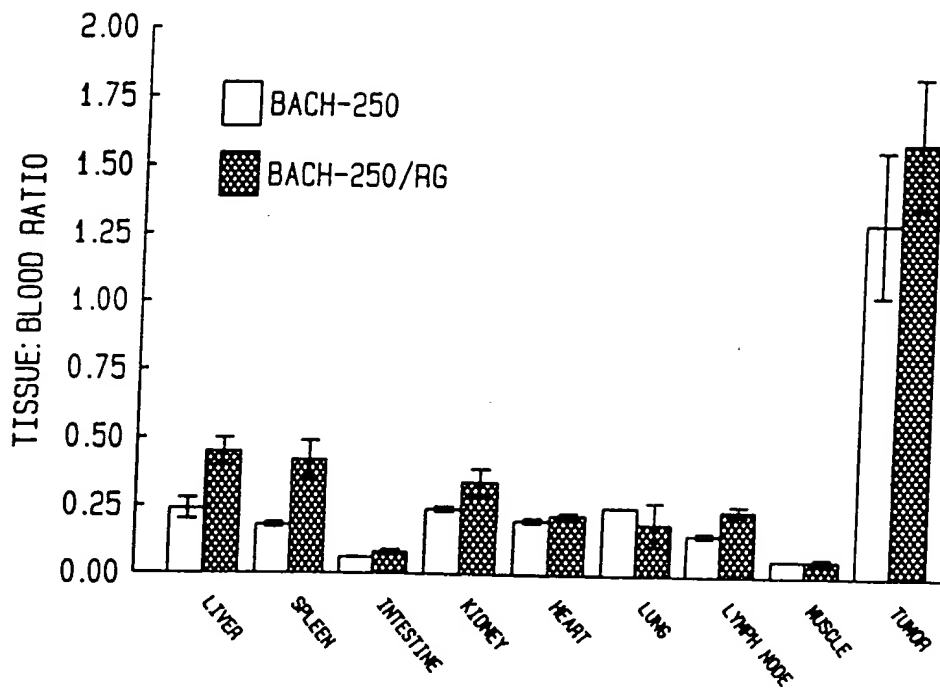


Figure 20

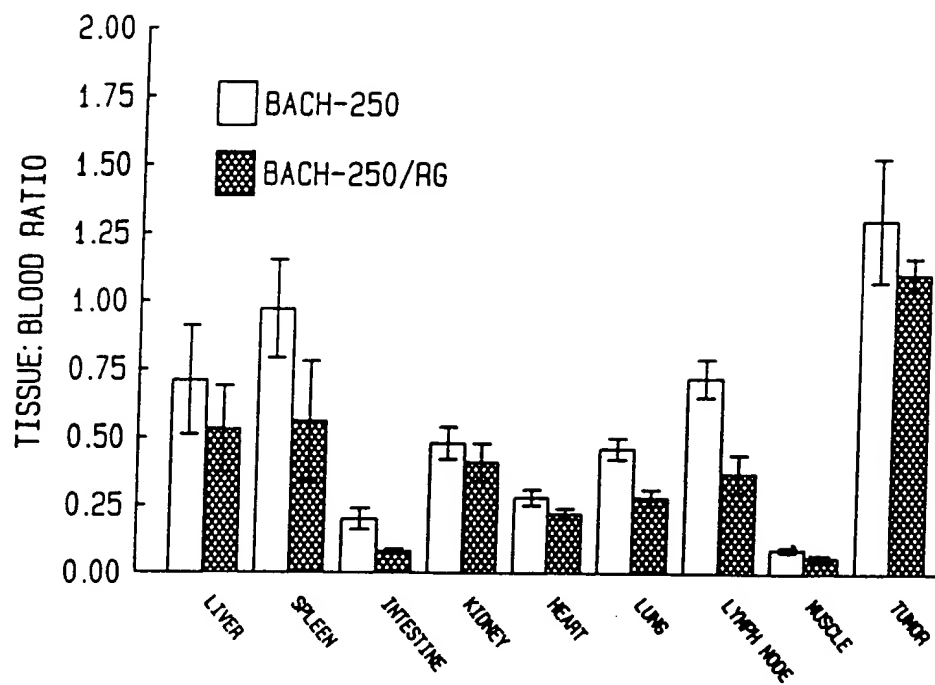


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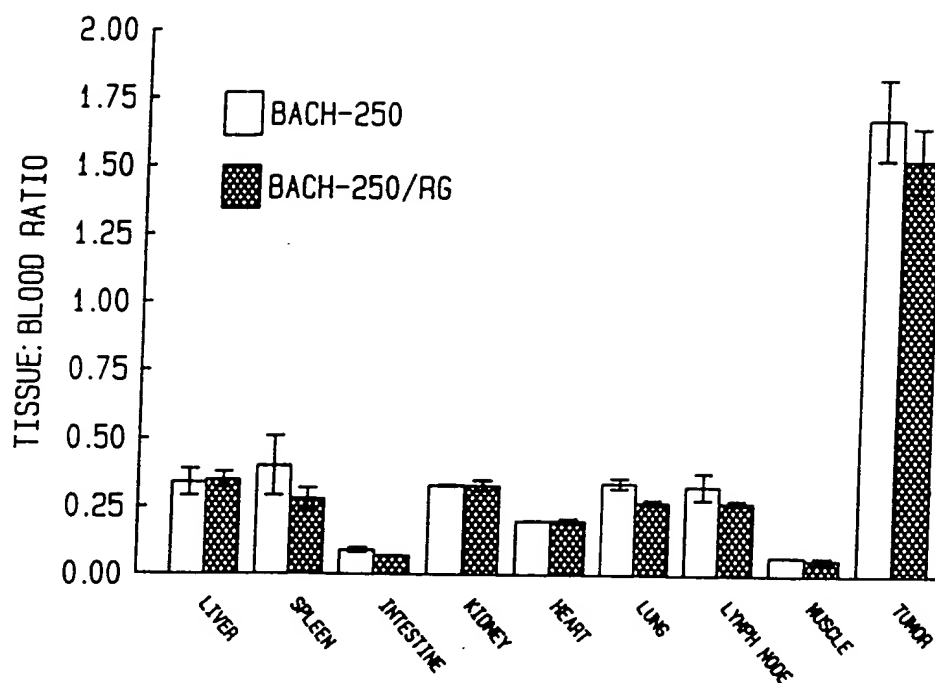


Figure 22

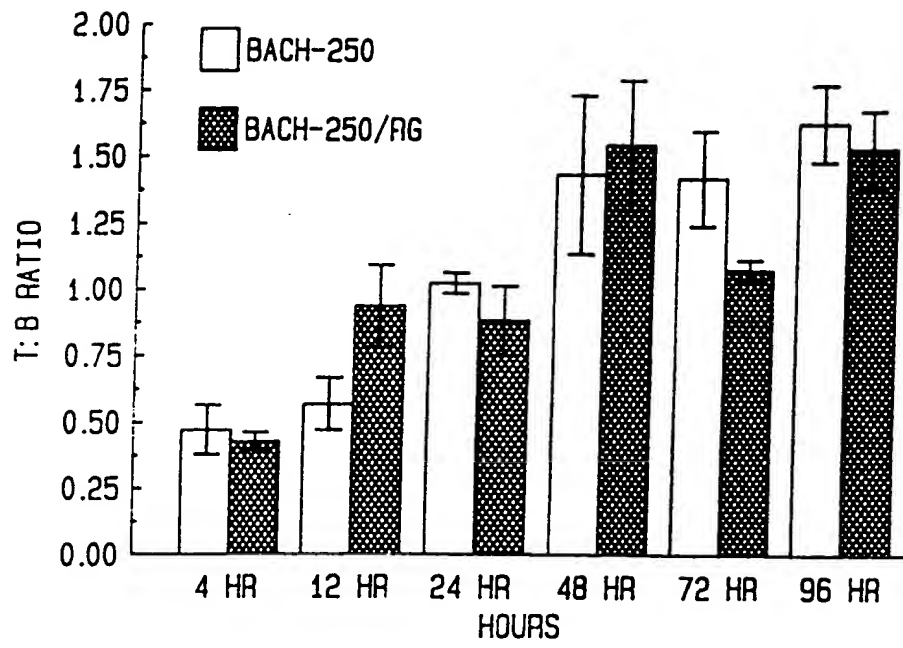


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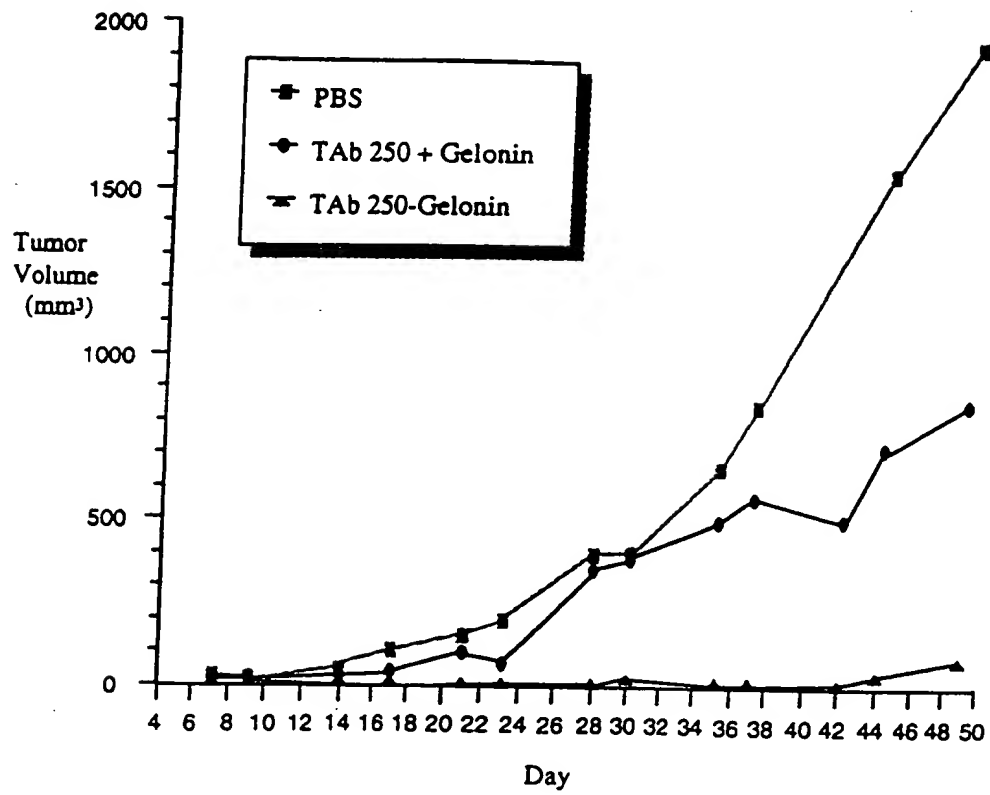


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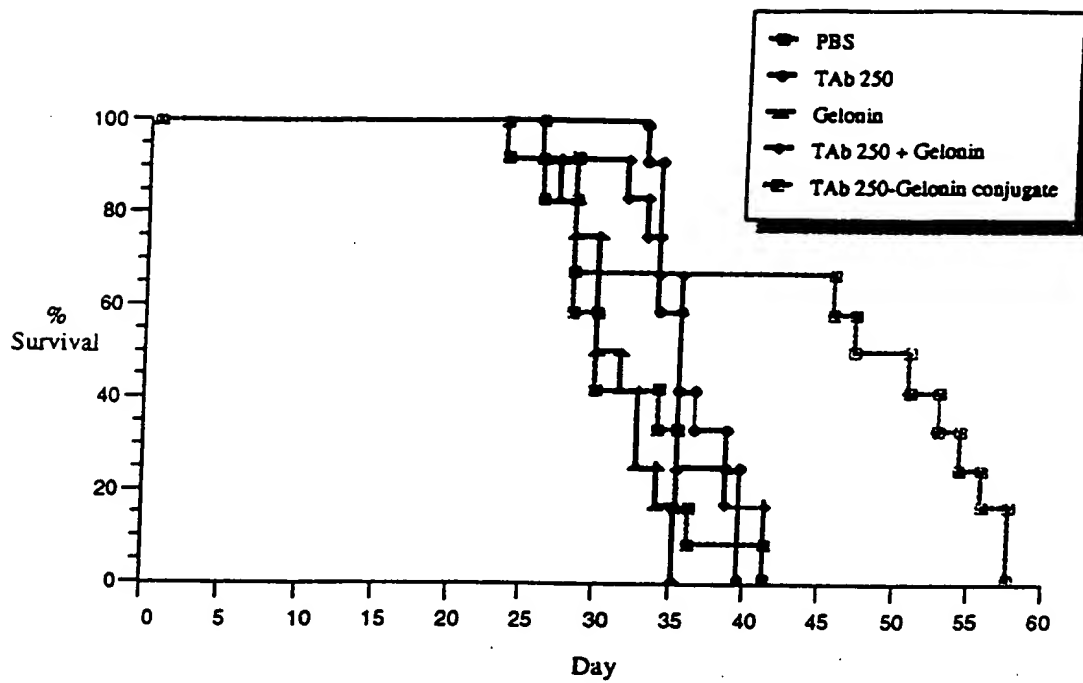




FIGURE 2 25

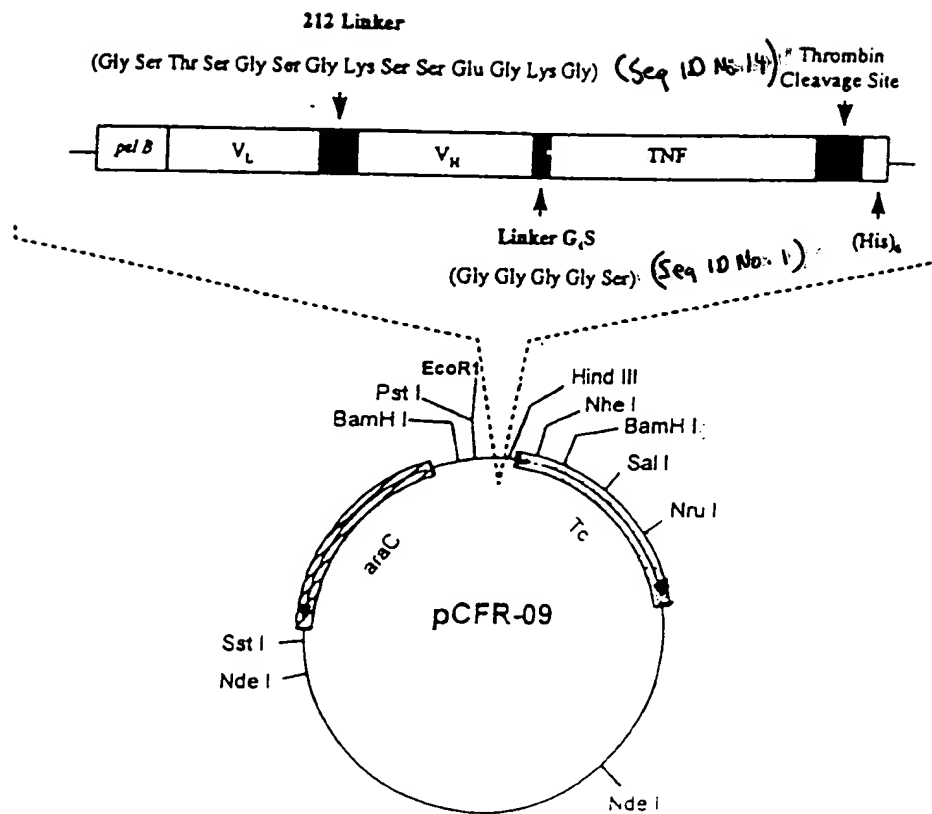


FIGURE 26

LANE      SAMPLE

- A    TNF(17kD) Standard
- B    Uninduced sFv23 bacterial lysate
- C    Induced sFv23 soluble lysate
- D    Affinity (IMAC) resin prior to elution
- E    sFv23 eluate from affinity resin
- F    Uninduced sFv23-TNF bacterial lysate
- G    Induced sFv23-TNF soluble lysate
- H    Affinity (IMAC) resin prior to elution
- I    sFv23-TNF conjugate from affinity resin
- J    Molecular weight markers

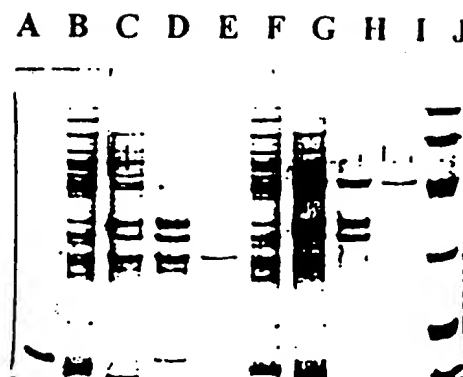
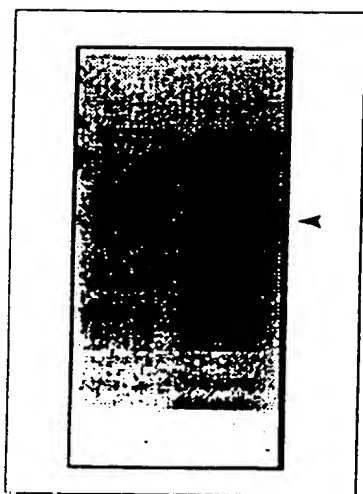
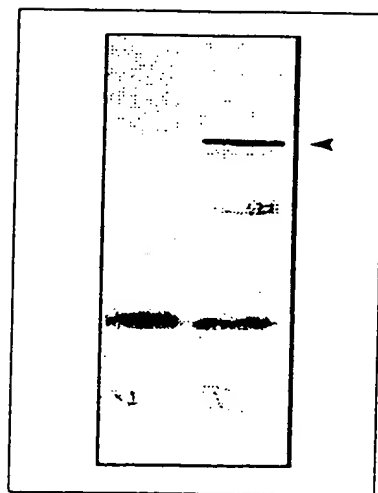


FIGURE 27

Coomassie



Anti-sfv23 Ab



Anti-TNF Ab

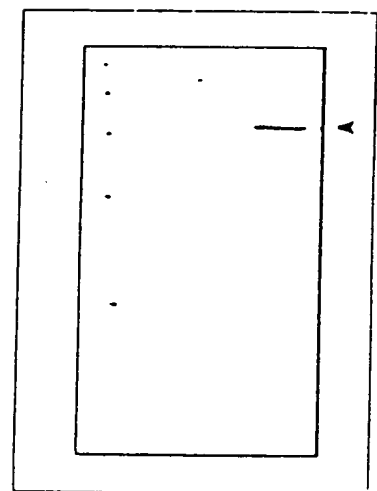


FIGURE \* 28

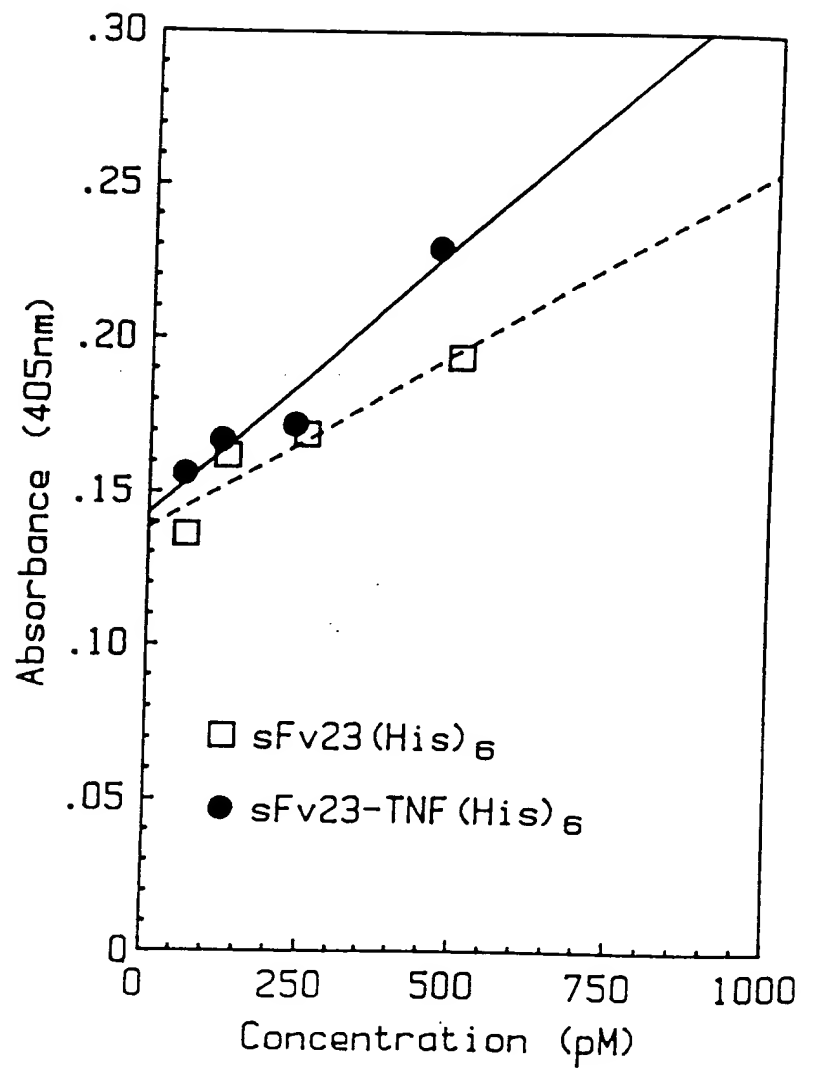


FIGURE 2 29

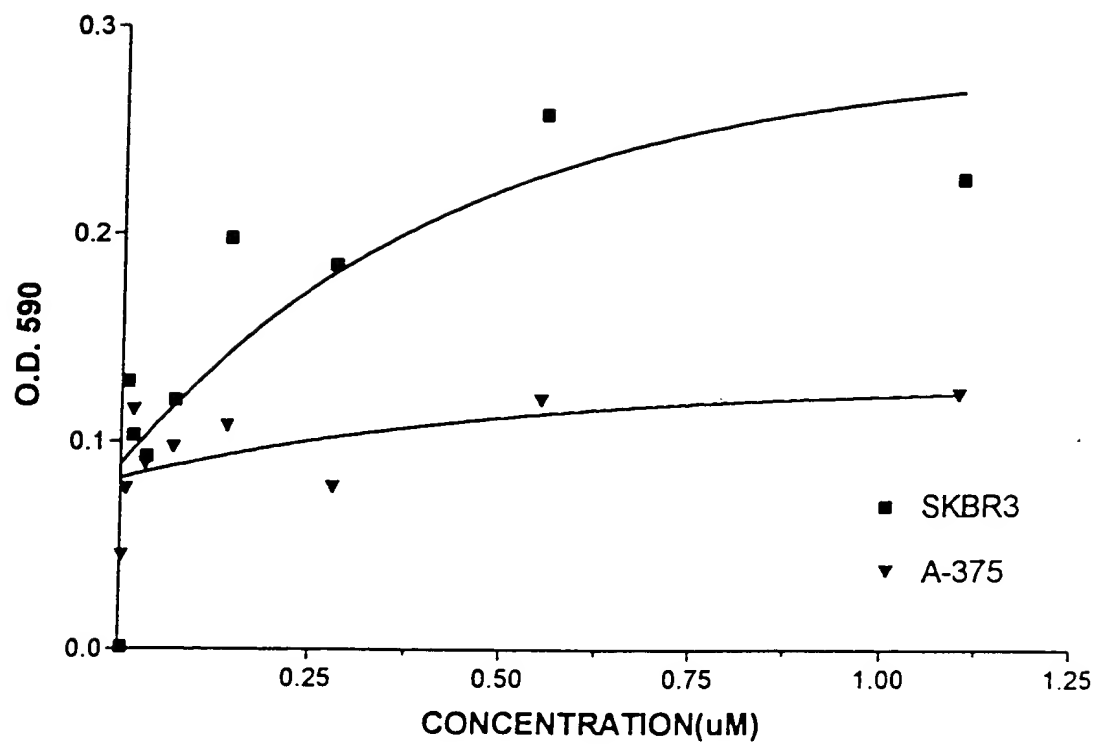


FIGURE 6 30

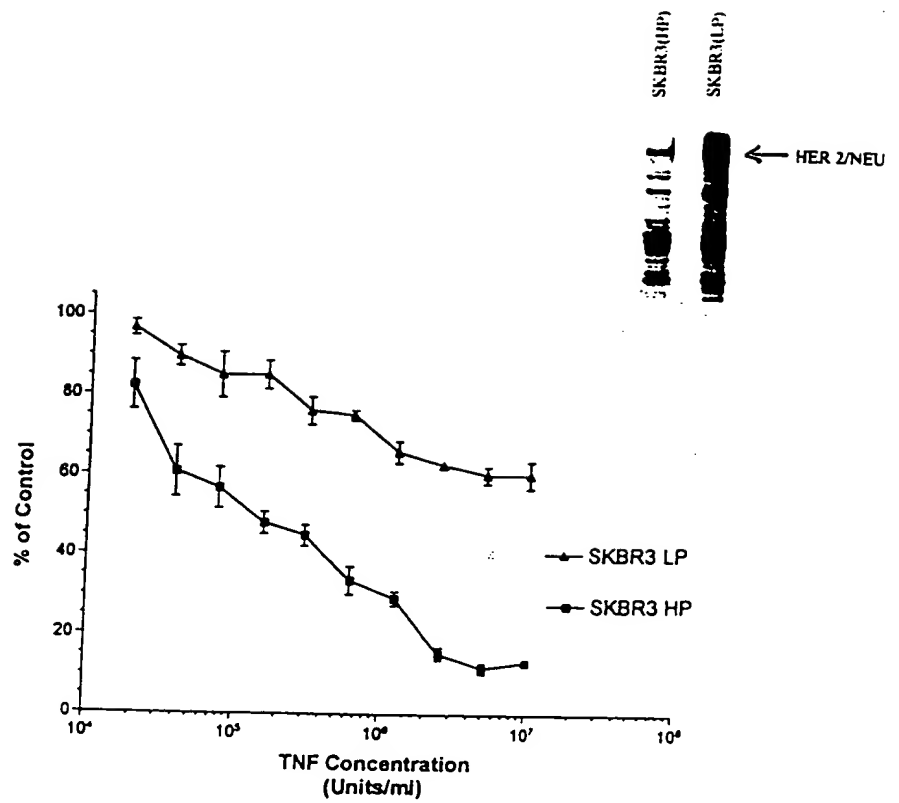


FIGURE 31

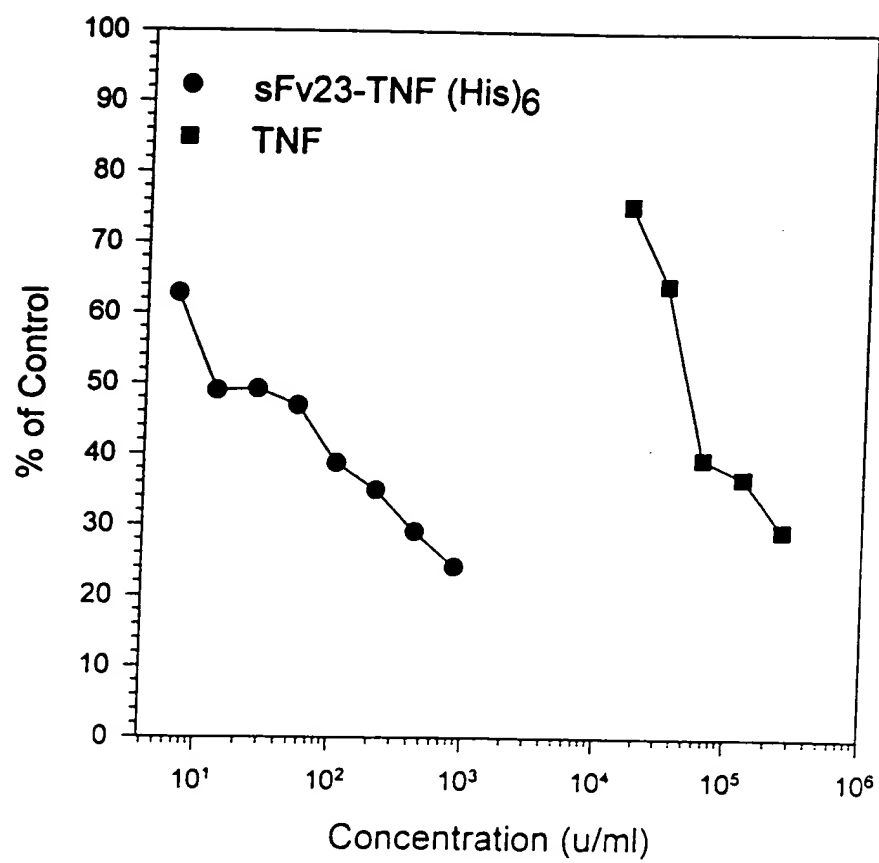
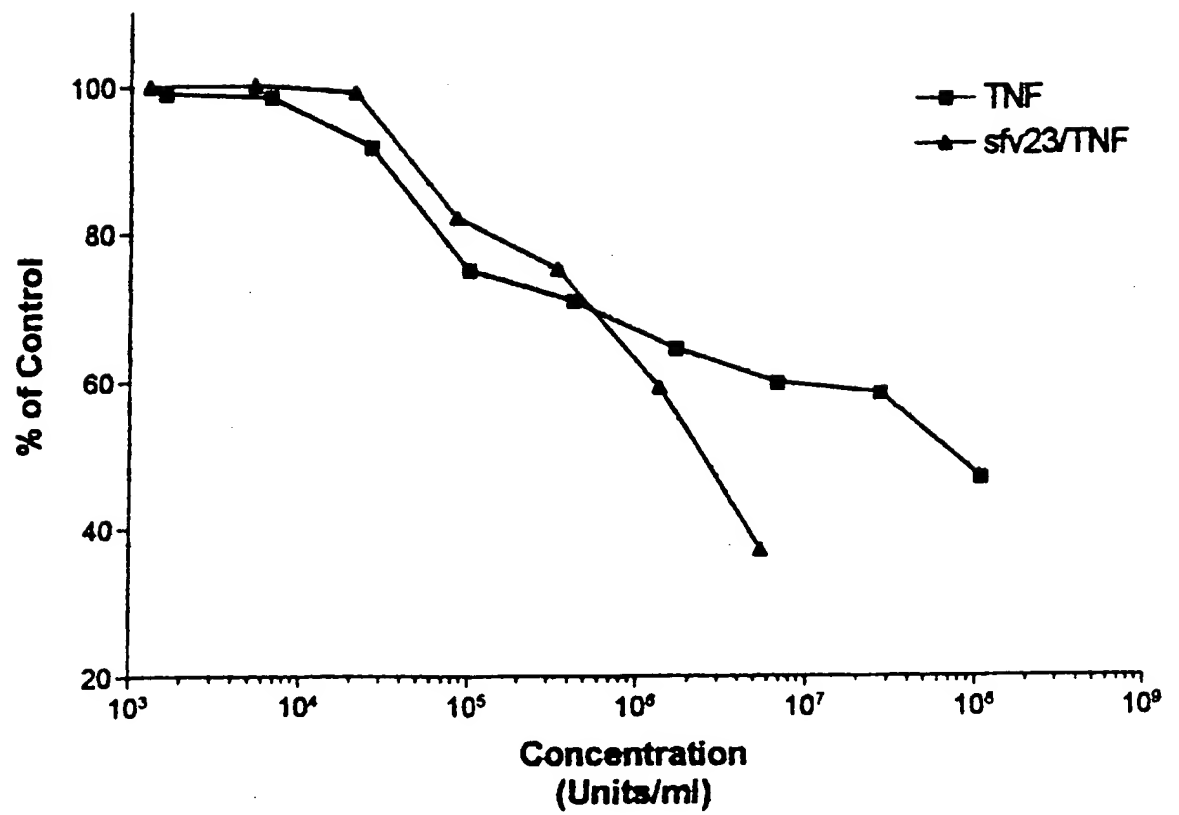


FIGURE 32





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